A RE-EXAMINATION OF THE OSMOTIC PROPERTIES OF THE PACIFIC HAGFISH, POLISTOTREMA STOUTI

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Investigations of various constituents of the blood of vertebrates have led to generalizations about vertebrate origins and relationships. The entirely marine myxinoids or hagfishes are morphologically primitive in the vertebrate assemblage and have unique osmotic and ionic properties. Most authors have concluded that hagfishes are approximately isotonic to their environment and that the osmotic concentration of the blood is made up largely of electrolytes. In some instances results have shown either a slight or marked hypertonicity of the blood serum with respect to the environment (summary in Table I), in contrast to the general conclusion of isotonicity.

Dekhuyzen (1904), Greene (1904) and the Schmidt-Nielsens (1923) concluded from their measurements of the freezing-point depressions of the blood sera and external medium that hagfishes must be isotonic. Smith (1932) interpreted his results, and those of Dekhuyzen and of Greene, however, to indicate a slight hypertonicity. Borei (1935), in contrast, concluded that *Myxine* was distinctly hypotonic to its environment. Krogh (1939, p. 119) considered that the blood of hagfishes is in almost complete osmotic equilibrium with sea water. In the most recent review, Black (1957, p. 187) concurs with Krogh, but her Figure 4 suggests that hagfishes are slightly hypertonic to sea water.

In view of the osmotic uniqueness of hagfishes it is important to know whether they are isotonic or are hypertonic to their environment. In the present investigation we have undertaken to determine whether the blood of the Pacific hagfish, *Polistotrema stouti,* is isotonic or hypertonic to the external medium over a range of concentrations. The chloride and sodium concentration of the blood serum and of the external environment were determined for comparison with previous work (see Table I). Since some of the variation of previous results may have been caused by different methods of handling and analysis, our own methods are described fully.

Materials and Methods

1. *Capture of animals*

On May 14, 1957, *Polistotrema stouti* (Lockington) was trapped by one of us (WNMcF) near San Diego, California. The trap was constructed from a 5-gallon can (MacGinitie and MacGinitie, 1949, p. 415), baited with two dead mackerel and set for 7 hours in 150 fathoms of water 4 miles north of North Coronado Island, Mexico. Of approximately 100 hagfish caught, 45 were transported to the laboratory at Marineland of the Pacific, Palos Verdes, California.

2. *Care of animals*

The hagfish were kept in three-gallon jars that were submerged in sea water in a large refrigerated tank (11–14° C.). Aerators were inserted through holes in the jar lids. There were no deaths under these conditions during a two-month period. Food was not offered until after the conclusion of the experiment performed on May 27. Dead mackerel were then placed in the jars on several occasions. Feeding was infrequent and the viscera only were consumed.

TABLE I

Previous measurements of osmotic pressure and chloride concentration of hagfish blood

Species	External medium		Internal medium		Internal medium[b] / External medium	Urea (mM/l.)	Source
	Δ_e[a] (° C.)	Chloride (milli-eq./l.)	Δ_i[a] (° C.)	Chloride (milli-eq./l.)			
M. glutinosa	1.73	—	1.74 1.83	—	Isotonic	—	Dekhuyzen (1904)
P. stouti	1.92	—	1.97	—	Isotonic	—	Greene (1904)
M. glutinosa	0.97 1.26 1.85 2.32	—	1.25 1.40 1.85 2.32	—	Isotonic	—	Schmidt-Nielsens (1923)
M. glutinosa	1.88	—	1.85 1.93 1.98	465 to 476	Hypertonic	2–4	Smith (1932)
P. stouti	—	384 467 530 626	—	344 414 471 570	Isotonic Cl_i/Cl_e = 0.89	Not detected	Bond, Cary and Hutchinson (1932)
M. glutinosa	1.90[c]	520	1.50[c]	325[d]	Hypotonic Cl_i/Cl_e = 0.62	58–62	Borei (1935)
M. glutinosa	—	483	—	448	Isotonic Cl_i/Cl_e = 0.93	—	Cole (1940)[e]
M. glutinosa	—	592[f]	—	576[f]	Isotonic Cl_i/Cl_e = 0.91[f]	2–4	Robertson (1954)

[a] Subscript e = external; i = internal.

[b] Interpretation of original author.

[c] Calculated from chloride and urea concentrations.

[d] Analysis of whole blood; low value may be related to low chloride concentration of red blood cells (see Robertson, 1954).

[e] Data of Smith, obtained in 1927.

[f] Chloride values are millieq./kg. water; ratio has been converted so that it is directly comparable to the others.

3. *Adjustment of animals to sea water of three different concentrations*

In addition to natural sea water (100%) a dilute medium of approximately 85% sea water was obtained by mixing 100% sea water and distilled water. Sea water was concentrated by boiling to half the original volume. This was mixed with natural sea water to provide a medium of approximately 115% sea water. The precise concentrations were obtained from freezing-point measurements. Hagfish were transferred in the three-gallon jars directly to sea water of these three concentrations. After an adjustment period of 30 hours, in which temperature was controlled by partially immersing the jars in the refrigerated tank, the hagfish were transported in these vessels to the University of California, Los Angeles, where the sampling was performed. Upon arrival the jars were placed in a cold room (2° C.) for a period of 1–3 hours. During this time the water temperature declined from 11.0° C. to a low value of 6.5° C. These temperatures are within the normal temperature range hagfish encounter.

4. *Blood sampling*

To facilitate withdrawal of blood samples the hagfish were stretched on a board with hemostats. Blood was removed in a syringe from the subcutaneous sinus. The freezing point of the first 0.2 ml. of whole blood was determined while more blood was withdrawn from the animal. Difficulty in obtaining blood from the thoracic or caudal hearts or from the systemic blood vessels made use of the sinus necessary.

The subcutaneous sinus of hagfishes is part of the blood circulatory system and not part of the lymphatic system (Cole, 1926, p. 322). It is one of a large system of sinuses which in cyclostomes appear to partially replace capillary beds. Blood flows into this extensive sinus from arteries of the snout and slowly moves posteriorly to the tip of the tail, where it is collected in a special vessel and pumped into the systemic circulation by the caudal hearts.

After withdrawal blood was placed in a 12-ml. centrifuge tube. A preliminary trial had shown that centrifugation of uncovered blood samples concentrated the blood sera by evaporation. The blood samples were therefore covered with paraffin oil immediately after withdrawal. No anticoagulant was necessary. Following centrifugation at 3500 times gravity for 20 minutes to remove cellular elements, the yellow or reddish supernatant fluid was drawn from beneath the paraffin oil in a slender-tipped pipette and placed in another centrifuge tube. Samples for chloride and sodium ion determination were removed and again a paraffin oil cover was added. The blood sampling and whole-blood freezing-point determinations were performed at the same time; freezing points of the serum were measured within the next 24 hours. The covered serum samples were stored in a refrigerator at 5° C.

The total length, weight and sex were determined for each specimen following blood removal. Specimens ranged in total length from 300 mm. to 460 mm. Their weights varied from 41.4 gm. to 135.3 gm.

5. *Freezing-point determinations*

Freezing points were measured with the Fiske Osmometer, which employs a thermistor. Samples of 0.2 ml. were placed in the small sample adapter of this

instrument and rapidly supercooled in the propylene glycol bath (− 10° C.). Vibration of a wire initiated freezing of the sample; resistance of the thermistor element, which was located in the freezing mixture, was balanced with a variable resistance. The osmometer had been previously calibrated with standard NaCl solutions; individual determinations of single samples varied no more than ± 0.02° C. Just before withdrawal of the hagfish from each container, freezing points of a water sample from that container were determined three times and the mean of these values recorded. Whole-blood and serum samples were determined only once to prevent denaturation of the protein fraction from changing the osmotic pressure of the frozen and thawed samples.

6. *Chloride and sodium analysis*

The chloride concentrations of the medium and serum were determined by a modification of the Volhard method reported by Keys (1937) and Consolazio, Johnson and Marek (1951). The small volume of blood obtained did not allow replicate determinations; each result therefore represents one titration. A mean error of 3.3% was obtained in analyses of standard NaCl soluitons. Chloride values are reported in milliequivalents per liter. Sodium was determined with flame photometry by the direct method. A Beckman Model B spectrophotometer with flame attachment was used in this analysis. A standard curve was established from a solution containing sodium, potassium, calcium and magnesium in the proportions reported for these elements in sea water of 19 parts per thousand chlorinity (Sverdrup, Johnson and Fleming, 1942, p. 186). No special provision was made to remove proteins prior to analysis, nor was any agent, such as isopropyl alcohol, used in the diluting solution to aid in ignition. The mean error determined was 3.2%.

Results

Osmotic data

Experiment 1

Jars of 85.5%, 100%, and 116.1% sea water (percentages based on freezing-point determinations), each containing three hagfish which had been kept at these concentrations for 30 hours, were brought to the laboratory on May 20, 1957. Freezing-point depressions of the external medium and of whole blood and serum samples of each hagfish were measured (Table II). Initially blood was drawn from one animal at each concentration (specimens 1D, 1N and 1C, Table II) and the osmotic pressure determined. The second animals tested at each sea water concentration (2D, 2N and 2C) had higher osmotic pressures. One hour was allowed to pass before specimens 3D, 3N, and 3C were removed and the blood samples obtained. The osmotic pressure of these last three hagfish returned to values near the concentrations of their respective environments.

This experiment indicated that *Polistotrema stouti* becomes isotonic or slightly hypertonic to its environment over the range of salinities from $\Delta = 1.59$ to 2.16° C. The Δ's of serum and whole blood were almost the same (ratio of Δ_s/Δ_{wb} from 99.4% to 102.3%). The slight hypertonicity of whole blood and serum to the external medium appeared to increase during the experiment, but after an hour the

blood returned toward isotonicity with the environment. These changes were quite uniform throughout the range of salinities tested.

Experiment 2

A second experiment was performed on May 27 with sea water at 4 concentrations from 83.5% ($\Delta = 1.57°$ C.) to 116.0% ($\Delta = 2.18°$ C.). Only a single hagfish was kept in each jar. The freezing point of the sea water in each container was measured just before removal of the hagfish.

TABLE II

Freezing-point depressions of whole blood (Δ_{wb}) and serum (Δ_s) of hagfish which had been kept in 3 different concentrations (Δ_e) of sea water for 30 hours. D, N, and C refer to dilute (85%), normal (100%) and concentrated (116%) media

	Sea water concentration (%)	Δ_e (° C.)	Animal number	Δ_{wb} (° C.)	Δ_s (° C.)	$\frac{\Delta_{wb}}{\Delta_e}$ (%)	$\frac{\Delta_s}{\Delta_e}$ (%)	$\frac{\Delta_s}{\Delta_{wb}}$ (%)
Experiment 1	85.5	1.59	1D	1.59	1.58	100.0	99.4	99.4
			2D	1.64	1.65	103.1	103.8	100.6
			3D[a]	1.61	1.60	101.3	100.6	99.4
	100.0	1.86	1N	1.87	1.89	100.5	101.6	101.1
			2N	1.93	1.94	103.8	104.3	100.5
			3N[a]	1.88	1.89	101.1	101.6	100.5
	116.1	2.16	1C	2.21	2.21	102.3	102.3	100.0
			2C	2.22	2.27	102.8	105.1	102.3
			3C[a]	2.19	2.23	101.4	103.2	101.8
Experiment 2[b]	84.6	1.59	4D	1.59	1.59	100.0	100.0	100.0
	83.5	1.57	5D	1.58	1.57	100.6	100.0	99.4
	100.0	1.88	4N	1.89	1.90	100.5	101.1	100.5
	100.0	1.88	5N	1.91	1.88	101.6	100.0	98.4
	105.9	1.99	4C	1.96	2.00	98.5	100.5	102.0
	116.0	2.18	5C	2.19	2.22	100.5	101.8	101.4

[a] A one-hour wait intervened before blood samples were drawn from the last animal in each container.

[b] Each hagfish kept in separate container.

The results (Table II) leave no doubt that the hagfish under these conditions were isotonic, not hypertonic, to the external medium (Δ_e between 1.57 and 2.18° C.). The Δ of 1.99° C. in one of the jars of concentrated sea water was the result of an unintentional dilution with 100% sea water during the preliminary adjustment period. The individual at this concentration, however, matched its osmotic environment about as well as the others.

In both experiments animals at different concentrations showed perceptible differences in the available volume and the apparent viscosity of blood. Those in diluted sea water provided a greater quantity of more "watery" blood than normal.

In concentrated sea water smaller amounts of more viscous blood were obtained. The external appearance of the animals did not differ appreciably in the three sea water concentrations.

Ionic data

The serum chloride and sodium concentrations corresponding to the freezing points reported in Experiments 1 and 2 are listed in Table III. Like the freezing-point values, the serum chlorides of the hagfish in Experiment 1 also show an

TABLE III

Sodium and chloride concentrations of the serum of Polistotrema stouti in Experiments 1 and 2

	Animal number	Total concentration[a] (milliosmols)		Milliequivalents/liter			
		Medium	Blood serum	Cl_e	Cl_i	Na_e	Na_i
Experiment 1	1D	853	849	441	419	330	—
	2D	853	886	441	431	330	360
	3D	853	861	441	385	330	335
	1N	999	1018	514	490	480	450
	2N	999	1043	514	516	—	—
	3N	999	1015	514	487	480	405
	1C	1162	1187	619	549	590	520
	2C	1162	1220	619	538	590	520
	3C	1162	1200	619	472	—	—
Experiment 2	4D	855	855	449	370	360	415
	5D	847	845	450	435	—	—
	4N	1010	1023	469	431	390	430
	5N	1010	1012	480	492	—	—
	4C[b]	1071	1077	530	502	405	455
	5C	1172	1193	576	529	570	475

[a] Determined from freezing-point depression.
[b] 106% sea water.

increase in concentration in the second animals tested (specimens 2D, 2N, and 2C) at each concentration. One hour later the hagfish (3D, 3N, 3C) showed a decline in serum chloride toward or below the original value. Where data were obtained, the sodium concentrations of sera in Experiment 1 also exhibit this trend.

DISCUSSION

From the experiments described above it is clear that the Pacific hagfish, *Polistotrema stouti*, is isotonic to the external medium, given time (30 hours in these experiments) to adjust. A slight apparent hypertonicity (no more than 5%) which developed during the course of Experiment 1 was eliminated in Ex-

periment 2 by keeping the hagfish separate. In Experiment 2 the average of $\Delta_{wb}/\Delta_e = 100.3\%$ and of $\Delta_s/\Delta_e = 100.7\%$. These values indicate complete isotonicity, within experimental error of the methods used.

Comparison of Experiments 1 and 2 offers a possible explanation for some of the differences reported by earlier workers. In handling the hagfish prior to withdrawal of blood it was impossible to prevent them from secreting slime copiously. In Experiment 1 removal of the first animal from each jar disturbed the others and caused sliming. The blood of the second animal from each medium was more concentrated than the first, but after an hour's wait the third animal from each medium was nearly isotonic. It seemed possible, therefore, that sliming induced a temporary hypertonicity of the blood. Support for this hypothesis was obtained in a subsequent experiment, in which as many as four small blood samples were withdrawn from single individuals at different times. Initial blood samples indicated isotonicity with sea water; production of slime was followed by hypertonicity (freezing-point measurements of whole blood samples) of 1–3%, which lasted an

TABLE IV

The contribution of sodium and chloride ions to the osmotic pressure of Polistotrema stouti. Ratios are mean values computed from the data of Table III. Parentheses denote the number of determinations from which each mean was computed

Concentration of medium (%)	Cl_i/Cl_e	Na_i/Na_e	$\frac{Na_e + Cl_e}{\text{milliosmols}_e}$[a]	$\frac{Na_i + Cl_i}{\text{milliosmols}_i}$[a]
85	0.918(5)	1.088(3)	0.914(4)	0.882(3)
100	0.970(5)	0.952(3)	0.946(3)	0.881(3)
116	0.874(5)	0.914(4)	0.985(4)	0.874(4)
Grand mean	0.918(15)	0.965(10)	0.953(11)	0.878(10)

[a] Determined from freezing-point depression.

hour or more. Much greater increases in the blood concentration were induced by rough handling or by placing the hagfish in a dry atmosphere. After a combination of these treatments for 10 minutes, the blood became 15–25% hypertonic to sea water. Osmotic recovery was nearly complete within 24 hours. The slight hypertonicity of hagfish blood indicated in the results of Dekhuyzen (1904), Greene (1904) and Smith (1932) may have been caused by handling or the secretion of slime. This could not account for the hypotonic values calculated by Borei (1935).

Under conditions of the present experiments hagfish become isotonic to 84–116% sea water ($\Delta_e = 1.57$ to 2.18° C.). The limits of their ability to endure changed external concentrations were not tested and it is not clear whether they might be able to regulate their osmotic concentration in some range other than 84–116% sea water. An insufficient number of hagfish was obtained to investigate this problem; work will be continued as more animals become available. The mean value (15 animals) of Cl_i/Cl_e was 0.92, which is similar to reports of most other authors (see Table I). In Robertson's paper (1954) on *Myxine* the sum of Cl_i and Na_i values accounted for 95.9% of the total osmotic concentration of the blood. In the present work this ratio was 95.3%, showing the close agree-

ment of our results with his. For computation of this ratio the grand mean of $Na_i + Cl_i$/milliosmols$_i$ (Table IV) was divided by 0.921, the factor given by Robertson to convert molar to molal concentrations in *Myxine*.

About 90% of the total osmotic concentration of the serum is due to sodium and chloride ions (Table IV); Robertson (1954) showed that other ionic constituents account for most of the remaining concentration. Both he and Smith (1932) found that there were 2–4 mM/l. of urea in the blood of *Myxine*. The high values of 58–62 mM/l. obtained by Borei (1935) are probably incorrect (nonspecific tests were performed on whole blood samples; see Robertson, 1954). In a personal communication Dr. Ernest Baldwin informs us that he has not been able to detect urea in the liver of *Polistotrema*. He points out that there is little likelihood that it would occur elsewhere in the body when absent from the liver. Dr. Baldwin, like Black (1957, p. 182), suggests that the nature of the food would affect the urea content of the tissues. It is possible that even the very low concentrations of urea reported by Robertson and Smith had an exogenous origin. Urea cannot have an importance in the osmotic composition of hagfishes comparable to that which it has in elasmobranchs.

Although the hagfishes resemble other vertebrates in the ratios of the ionic constituents of their blood (Cole, 1940; Robertson, 1954), they are unique among vertebrates in being isotonic to sea water and having the osmotic concentration of the blood composed almost entirely of ionic constituents. Hagfishes are poikilosmotic within the range of sea water concentrations investigated, which encompass those of the marine habitats they normally occupy.

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Summary

1. Determinations of the osmotic pressure of whole blood and serum show that *Polistotrema stouti* is isotonic in sea water concentrations from 85 to 116%.

2. Hypertonicity of the blood can be induced experimentally by disturbance of the animals. This factor could account for hypertonic values reported previously.

3. Serum sodium and chloride account for 88% of the total osmotic pressure. The mean Cl_i/Cl_e ratio equals 0.92. The hypertonicity which can be produced by disturbance is reflected by a rise in the serum sodium and chloride concentrations.

4. Urea is absent from the liver and is considered to have no significance in the osmotic composition.

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